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The Role of the Immune System in Conjunctival Wound Healing After Glaucoma Surgery

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Abstract. The immune system has a fundamental role in the development and regulation of ocular healing, which plays an important role in the pathogenesis of most blinding diseases. This review discusses the mechanisms of normal wound healing, describing the animal and fetal wound healing models used to provide further insight into normal wound repair. In particular, conjunctival wound repair after glaucoma filtration surgery will be used to illustrate the contributions that the different components of the immune system make to the healing process. The potential role of macrophages, the possible regulatory effect of lymphocytes, and the important role of growth factors and cytokines in the wound healing reaction are discussed. The significance of the immune system in the pathogenesis of aggressive conjunctival scarring is addressed, particularly assessing the predisposing factors, including drugs, age, and ethnicity. The rationale behind the pharmacological agents currently used to modulate the wound healing response and the effects these drugs have on the function of the immune system are described. Finally, potential new therapeutic approaches to regulating the wound healing response are reported. (**Surv Ophthalmol 45**:49–68, 2000. © 2000 by Elsevier Science Inc. All rights reserved.)

Key words. anti-metabolites • conjunctival wound healing • corticosteroids • fibroblasts • glaucoma surgery • immune system • macrophages • T lymphocytes

In discussions of conjunctival wound healing, the fibroblast has received the most attention. The purpose of this review is to describe the fundamental and complex role of the immune system in normal conjunctival wound repair and to indicate how it may contribute to excessive wound healing.

The modulation of abnormal conjunctival wound healing is a subject of great interest, because excessive subconjunctival scarring is the main reason for the failure of glaucoma filtration surgery.[2] As the successful surgical treatment of glaucoma is probably the most effective way to preserve vision, and indeed, in many parts of the world is often the only practical treatment available, the ability to control the wound healing re-

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sponse to maximize the success rate of glaucoma filtration surgery is vitally important.[130,164] In addition, it is important to understand why some filtration operations fail despite the use of antifibrotic agents.

I. Wound Healing Repair Models

A. ANIMAL MODELS

Healthy conjunctiva is normally populated with cells belonging to the immune system.[9,56,208] T lymphocytes, macrophages, Langerhans' cells, and occasional B cells have been identified in the epithelium and substantia propria of conjunctival biopsies taken from patients without any primary conjunctival disease. The predominant lymphocyte is the T lymphocyte, with an estimated normal CD4:CD8 T cell ratio of approximately 1:2. During wound healing, there is a rapid and significant increase in inflammatory cell numbers.[199]

Various animal models have been used to study conjunctival scarring after glaucoma filtration surgery. We have learned from this work that the immune system must play an important role during the early stages of wound healing.[166,199] These models show the influx of inflammatory cells, including polymorphonuclear leukocytes, lymphocytes, and macrophages, into the injury site during the first few days following wounding (Fig. 1).

B. FETAL WOUND REPAIR

Further evidence that the immune system participates in wound healing comes from research into fetal wound repair.[161] Usually, during the first two trimesters of fetal development, fetal wound healing is not associated with an acute inflammatory reaction, and healing occurs without scar tissue formation.[5,117] Hopkinson-Woolley et al were not able to demonstrate a macrophage infiltrate in embryonic mouse excisional wounds.[124] However, after gestational day 14, macrophage recruitment did occur and this was coincident with the stage when fetal scarring began to develop. Interestingly, the addition of cytokines produced by macrophages, such as TGFβ or PDGF, to fetal wounds induces an inflammatory response and the development of scar tissue.[4,145]

II. The Inflammatory Phase of Wound Healing

A. ACTIVATION OF THE IMMUNE SYSTEM IN WOUND HEALING

The wound healing response can be subdivided into a sequence of specific events, made up of three stages: the inflammatory stage, the proliferative stage, and the maturation stage. The immune system

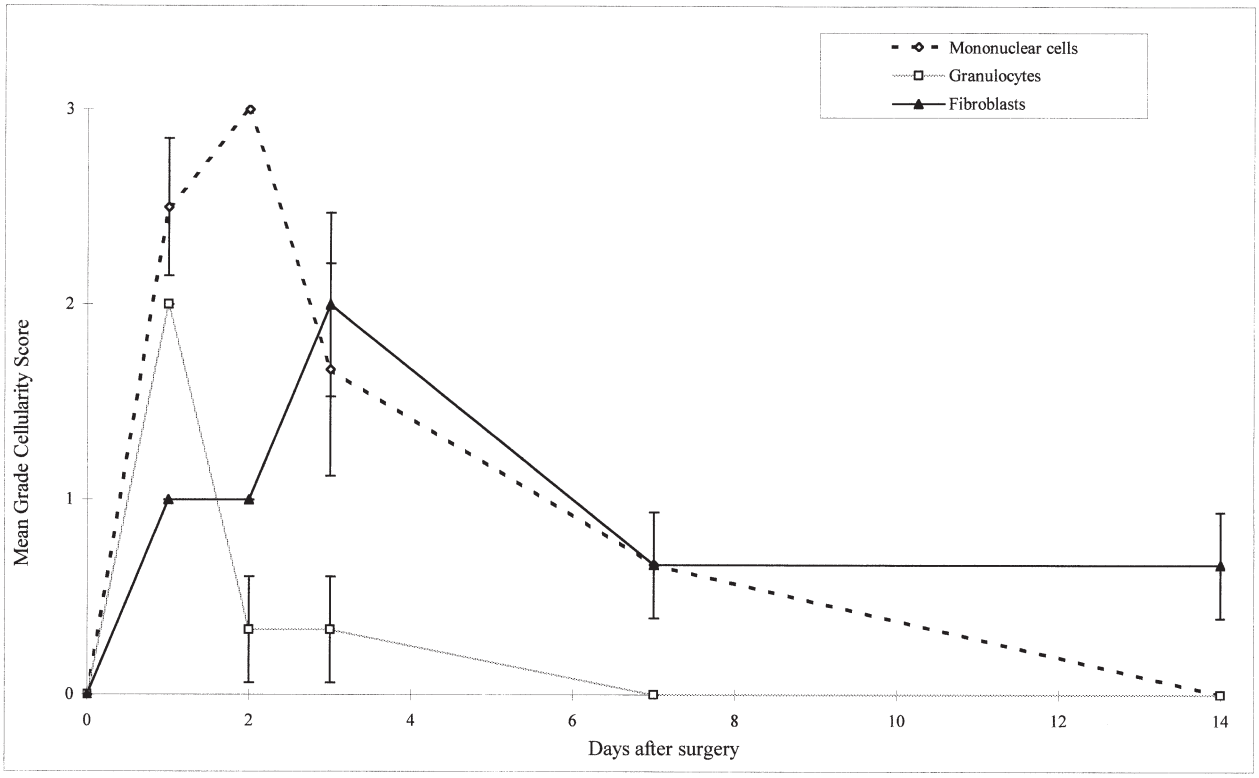

Fig. 1. The cellular profile of mononuclear cells, granulocytes and fibroblast activity, with consecutive peaks consistent with the classic wound healing response in a mouse model of conjunctival scarring. Error bars = S.E.M. (Courtesy of MF Cordeiro: The role of transforming growth factor beta in the conjunctival scarring response following glaucoma filtration surgery, PhD Thesis, University of London).

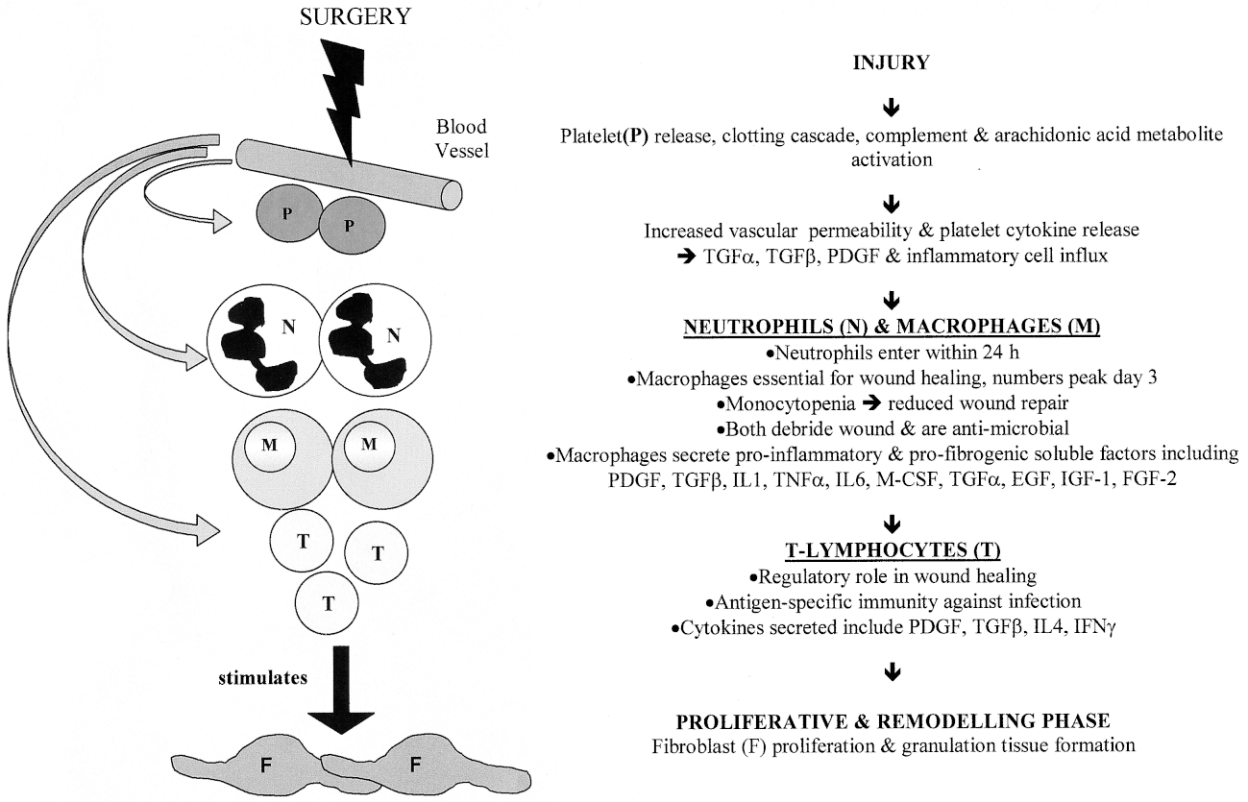

Fig. 2. The inflammatory stage of wound healing: a summary of the biochemical and cellular events in the inflammatory phase of wound healing.

is primarily active during the inflammatory stage, and this is summarized in Fig. 2.[21]

Initially, after tissue damage and blood vessel rupture, there is a release of blood cells and plasma proteins into the wound site. Hemostasis is achieved by vasoconstriction of the small blood vessels in the wounded area, which lasts for about 5–0 minutes. In addition, blood vessel rupture exposes subendothelial collagen, which stimulates platelet aggregation and activation of the intrinsic coagulation cascade, resulting in clot formation.[247] In 1 ml of peripheral blood there are normally 2–7 million neutrophils, 1–3 million lymphocytes and 0.2–0.8 million monocytes, so it is inevitable that the bleeding accompanying the beginning of an operation will introduce a large number of inflammatory cells into the wounded area. Indeed, the importance of hemostasis during glaucoma filtration surgery to avoid promoting excessive scarring postoperatively is well recognized.

Components of the clotting cascade, in particular Hageman factor, activate the innate immune system and stimulate the generation of the vasoactive peptide bradykinin.[134]

Platelets also release growth factors such as TGFα, TGFβ, and PDGF, which are chemotactic and mitogenic to inflammatory cells.[33,142] The degree of inflammation in a wound healing response is thought to be related to the concentration of cytokines initially released by platelets. Fetal porcine platelets contain lower quantities of the cytokines TGFβ and PDGF-AB, which may in part explain the reduced macrophage activation and inflammatory response associated with fetal wound repair.[178]

The innate immune system is the body's nonspecific early defense against invading pathogens. It is comprised of the complement cascade and phagocytic populations of neutrophils and macrophages, which clear the wound site of debris and infectious agents. The complement cascade plays a major role in the early events of conjunctival wound healing by initiating inflammation through amplifying the original injury signal and by stimulating the accumulation of mitogens and chemoattractants to the injury site.

Activated complement products, such as C3a and C5a, activate the prostaglandins and other components of arachidonic acid metabolism which stimulate an increase in vascular permeability.[105] They are chemotactic for phagocytic cells, such as neutrophils and monocytes. The combination of all these actions results in a further influx of inflammatory cells. Subsequently, the activated arachidonic acid metabo-

lites stimulate these inflammatory cells to release other vasoactive factors, such as leukotrienes, platelet activating factor, and histamine.[241] Complement products can also coat microbes so that they are more effectively taken up by the phagocytic cells in a process known as opsonization.

B. NEUTROPHILS IN WOUND HEALING

Neutrophils are the first inflammatory cells of the immune system to enter the wound area, accumulating within 6 hours and disappearing by the third day after wounding. They are attracted to the site of injury by chemoattractants, such as C5a, platelet factor 4 (PF4), leukotrienes, TGFβ, TNFα, IL1, and bacterial products.[40,79,108,233,238] Their movement into the extracellular matrix is helped by receptors on the vascular endothelium, called selectins, and integrin receptors on the neutrophils.[228]

The main function of these cells is to phagocytose bacteria and foreign material in the wound, and they may be important in clearing red blood cells from the wound site.[225] They may also be a source of proinflammatory cytokines such as IL1α, IL1β, and TNFα.[125] Although neutrophils are the first inflammatory cells to appear in wounds, they are not thought to be essential for normal wound repair, because if they are deficient and as long as an infection is not present, their function can be taken over by macrophages, and healing will occur normally.[225]

C. MACROPHAGES IN WOUND HEALING

The next inflammatory cells to enter the wound are monocytes, becoming the predominant inflammatory cell type at about 12 hours after injury.[129] They reach peak numbers on about the third day, but start to decrease by the fifth day. They are attracted to the wound area by chemoattractants, such as TGFβ and PF4, and are activated to become macrophages by factors released by platelets and phagocytosing substances, such as fibronectin and collagen.[32,79,246]

Unlike neutrophils, macrophages are essential for normal wound healing. This has been demonstrated by the studies of Leibovich and Ross, who used a combination of steroids and local antimacrophage serum to induce a systemic monocytopenia and to eliminate any local tissue macrophages. This deficiency produced a significant reduction in wound debridement and fibrogenesis in guinea pig skin wounds.[151] Also, reepithelialization was delayed, and fibroblasts did not begin to appear until the fifth day (in control wounds they were the predominant cell type by this day). They were located at the wound margins only and did not proliferate greatly.

Macrophages are important in recruiting and activating fibroblasts and other inflammatory cells. They produce numerous soluble factors that stimulate fibroblast proliferation, and when macrophages are injected into wounds they promote wound healing.[75,152] Moreover, corneal wound healing studies have shown that macrophages are potent stimulators of angiogenesis and collagen synthesis in a cell number dependent fashion.[127] Only one group of investigators has suggested that macrophages could act as negative regulators of fibroblast proliferation. Fukasawa et al demonstrated that the supernatant from cultured rabbit peritoneal macrophages was able to inhibit fibroblast proliferation; however, it is important to note that the fibroblasts were serum-deprived.[107] Therefore, in general, macrophages are thought of as stimulators of wound healing.

Macrophages produce enzymes such as collagenase and elastase, which debride the injury area and release anti-microbial factors, such as oxygen radicals and nitric oxide.[52,158] Importantly, they provide a link between the innate and specific parts of the immune system.

D. THE REGULATORY ROLE OF LYMPHOCYTES IN WOUND HEALING

It has long been known that lymphocytes are present in healing wounds.[81] Lymphocytes migrate into human skin wounds soon after the macrophages, appearing by the first day. Peak numbers develop between the eighth and fourteenth days after wounding and may persist for as long as 4 months.[160] In a rat wound-healing model, about 70% of the inflammatory cells were T cells by the tenth day after wounding.[43]

Lymphocytes comprise the body's specific immune response to injury and infection. T cells become activated when they recognize antigen presented to them by macrophages, subsequently resulting in the proliferation of antigen-specific T cells. The cytokines they release can activate additional immune cells in the vicinity, including other T cells, macrophages, and polymorphs in a process called *bystander activation.*

Both CD4+ (helper/effector) and CD8+ (cytotoxic) T cell subsets were found in a rat skin wound-healing model.[100] They first appeared from day 5 post-wounding, peaked on day 7 and persisted until day 10. In this model, there were a greater number of lymphocytes in the deeper aspects of wounds, and the CD4+T cells outnumbered the CD8+T cells in a 2:1 ratio. Although both subsets have been identified in the conjunctiva of glaucoma patients who have failed filtration surgery at 3 months, it is not yet fully known what role the different subsets may have in the development of normal and pathological scarring.[174] Fig. 3 summarizes the role of T lymphocytes in wound healing.

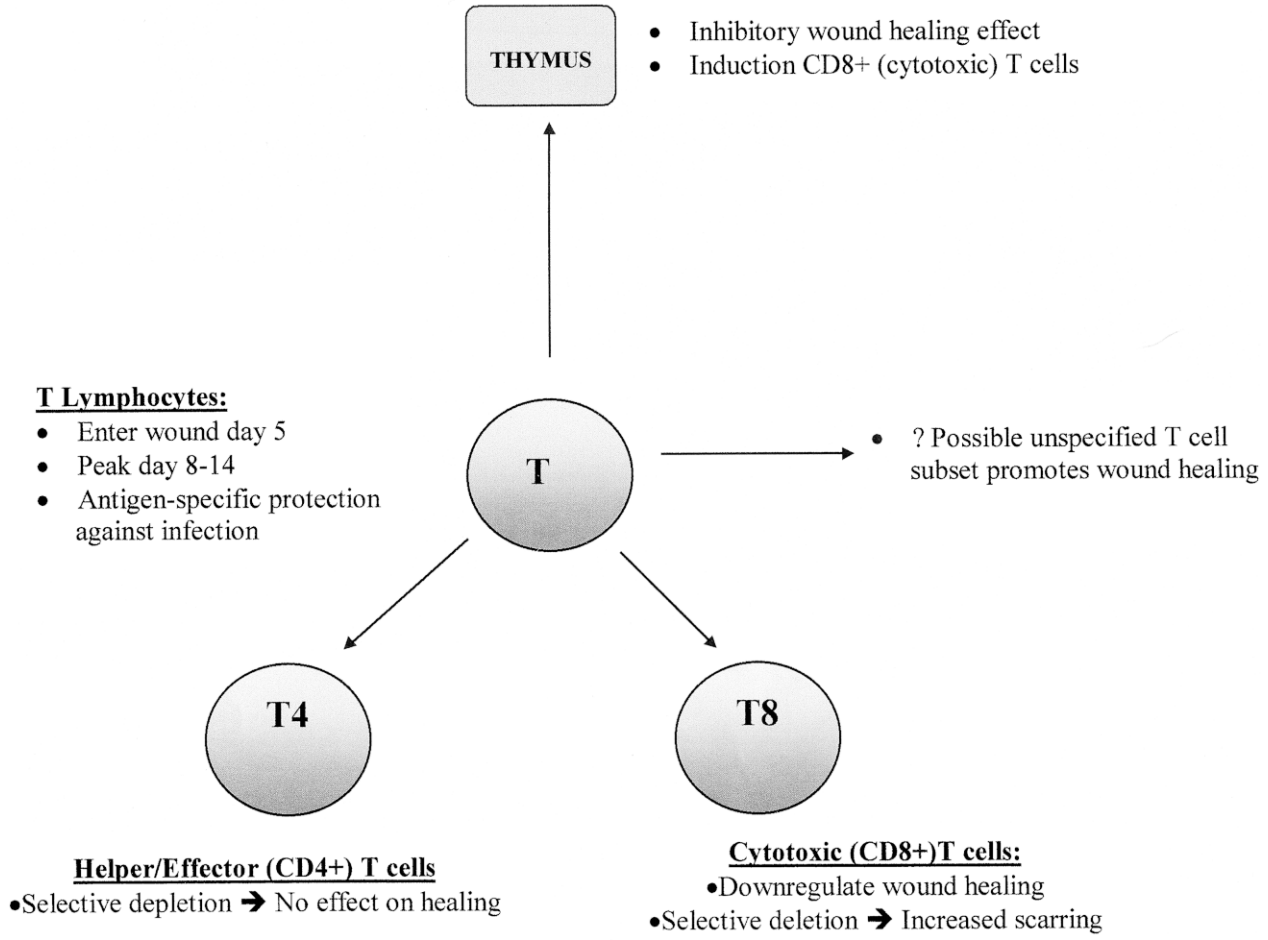

Fig. 3. The role of T lymphocytes in wound healing.

T cells are able to secrete factors that are either stimulatory or inhibitory to fibroblasts.[237,239] For instance, Neilson et al[171] and Postlethwaite et al[196] showed that, depending on culture conditions, lymphocytes produced a soluble factor, which either promoted or inhibited fibroblast collagen production. Breslin et al demonstrated that the mainly T cell infiltrate harvested from rat wounds on day 10 suppressed the growth of other T cells.[43] Furthermore, some researchers have found that activated T cells can inhibit fibroblast proliferation, possibly through the action of membrane-associated interferon-gamma.[62] Finally, activated T cells may affect fibroblast migration by stimulating increased expression of the proteolytic enzyme, matrix metalloproteinase-1 (MMP-1).[53,62] This could be important in enabling fibroblasts to contract the wound during the later stages of wound healing.

Other investigators have used experimental models that modulate lymphocyte function to demonstrate the regulatory role of lymphocytes in wound healing. Agents such as Vitamin A, arginine, and growth hormone, by stimulating T cell function, also increase collagen deposition and wound breaking strength.[22,86]

Conversely, the administration of agents such as steroids, retinoic acid, citral, and cyclosporin A, which all depress T cell function, are associated with impaired wound healing.[99]

It is thought that the thymus probably exerts an inhibitory effect on normal wound healing, perhaps by increasing T cell regulatory activity. T cells normally mature in the thymus, leaving it to enter the blood and go to peripheral lymphoid tissues. Thymectomized rats have a deficiency in CD8+T cell induction, and this is associated with an increased wound healing response attributable to greater collagen deposition. This can be reversed when thymic grafts are implanted or when T cells are reconstituted in the originally athymic mice.[24,25] When thymic hormones are administered, they stimulate a decrease in wound healing with a reduction in wound breaking strength and collagen deposition.[23]

Depletion studies have provided further insight into the possible regulatory role of lymphocytes in wound repair.[20,183] Firstly, global T cell depletion re-

sulted in impaired wound healing with a reduction in wound breaking strength and collagen deposition. Secondly, when the CD8+T cell subset was selectively removed, this produced a greatly increased wound healing reaction, suggesting a role for this subset in the down-regulation of wound repair. However, although simultaneous depletion of both CD4+ and CD8+T cells resulted in an increased wound healing reaction, when the CD4+T cell subset was selectively depleted, there was no change in wound healing. The authors suggested that there might exist an as yet unspecified T cell subset that could be responsible for promoting wound healing.

Recent research has focused on the existence of regulatory or anergic T cells, which may have a role in the prevention of autoimmune diseases.[55,106] These cells can actively inhibit the immune response of other T cells. There has been no research to investigate whether they have any function in regulating wound healing.

In summary, from the research to date, we surmise that T cells play a regulatory role in wound healing. They may start off by stimulating macrophages and fibroblasts, which could then be followed by the CD8+T cells in some way switching off wound repair. Questions remain regarding how the stimulatory and inhibitory factors interact to finely tune the healing response and what events signal the immune system to switch from a stimulatory to inhibitory role. We propose that the purpose of the immune system is not only to protect the individual from an infection while a wound resolves, but also to stimulate the development and regulate progress to the next stages of the wound healing reaction.

The majority of fibroblasts are derived from local tissue fibrocytes. However, recently a novel population of bloodborne fibroblast-like cells called *fibrocytes* has been described that may play a minor role in the earliest events following wounding.[51,60] They appear to make up about 0.5% of peripheral blood leukocytes, and enter the site of injury within 24 hours after wounding. In addition, these cells produce proinflammatory cytokines and chemokines, which may attract other inflammatory cells into the injury site. However, further research is still required to verify their possible role and contribution to wound repair.

III. Inflammatory Cytokines and Growth Factors in Wound Healing

Lymphocytes and macrophages exert many of their effects through the production of cytokines and growth factors. The research that has been conducted has used various techniques, including wound healing models to identify their presence in vivo and to study their effects on scarring when applied artificially, and the investigation of their effects on fibroblast function in vitro. Their actions are the subject of a number of reviews.[33,211] The following section deals with the individual cytokines researched and presents the evidence for their effects in wound healing (Table 1).

A. PLATELET-DERIVED GROWTH FACTOR (PDGF) AND TRANSFORMING GROWTH FACTOR BETA (TGFβ)

Two of the most important profibrogenic cytokines are PDGF and TGFβ, which are both produced by macrophages, lymphocytes, platelets, and fibroblasts, although the main producers of TGFβ are macrophages and fibroblasts.[16,17,136] Significant amounts of both TGFβ and PDGF have been detected in human wound fluids, with the highest concentration of PDGF developing immediately after surgery.[85]

PDGF is the most potent in vivo chemoattractant to other macrophages and fibroblasts, and it promotes increased granulation tissue formation when tested in vivo in a model of skin wound repair.[185,188,229] Although it does not directly stimulate fibroblast collagen synthesis, it does increase deposition of glycosaminoglycans and fibronectin.[189,190] PDGF probably exerts its effects indirectly in two ways: first, by increasing wound cellularity, and second, by inducing macrophages and fibroblasts to produce increased amounts of TGFβ. This in turn stimulates increased collagen synthesis.[187] It also stimulates fibroblast mitogenesis, including human Tenon's fibroblast proliferation in vitro.[92]

Mustoe et al in 1989 and Cromack et al in 1993 used models of impaired wound healing to investigate the actions of both PDGF and TGFβ.[67,169] First, total body irradiation was used to produce a monocytopenia with no concomitant effect on fibroblast numbers. This resulted in a significant decrease in wound breaking strength associated with a decrease in cellularity at the wound site. Topical PDGF wound application had no effect in improving wound breaking strength, even though it induced a local increase in fibroblast numbers; however, topical TGFβ did accelerate wound healing and increased wound strength. Second, megavolt electron beam irradiation of the skin was used to reduce skin fibroblast function, sparing bone marrow function. In contrast, the application of TGFβ had no effect on wound healing, but when PDGF was applied, there was an increase in wound strength with an associated increase in the number of macrophages and fibroblasts. These results suggest that macrophages are essential producers of PDGF, which mediates its wound healing effects indirectly by recruiting other

TABLE 1

The Cellular Sources and Effects of Growth Factors and Cytokines in Wound Healing

Cytokine	Cell Source	Cytokine Effects
TGFβ	• Platelets • Macrophages • T-lymphocytes • Fibroblasts	• Stimulates fibroblast migration, proliferation, and increased collagen synthesis • Chemoattractant to macrophages • Stimulates angiogenesis • Inhibits epithelial and endothelial migration and proliferation • TGF-β1, -β2, -β3 identified in mouse wound healing model
PDGF	• Platelets • Macrophages • T-lymphocytes • Fibroblasts • Epithelial cells • Endothelial cells • Smooth muscle cells	• Chemoattractant to macrophages and fibroblasts • Stimulates fibroblast, endothelial, and epithelial cell proliferation • Stimulates glycosaminoglycans and fibronectin production • Stimulates TGFβ secretion
TGFα and EGF	• Platelets • Macrophages • Epithelial cells	• Stimulates epithelial chemotaxis and proliferation • Stimulates angiogenesis • Stimulates fibroblast migration • Stimulates fibronectin synthesis
IGF-I	• Macrophages • T-lymphocytes • Epithelial cells • Endothelial cells • Fibroblasts • Smooth muscle cells	• Stimulates fibroblast migration, proliferation, ECM synthesis, and contraction • Stimulates angiogenesis • Stimulates epithelial cell migration and proliferation
TNFα	• Macrophages • T-lymphocytes	• Detected early in wound healing (first few days); probably pro-inflammatory • Synergistic with PDGF • Stimulates angiogenesis • Stimulates fibroblast secretion of M-CSF • Stimulates fibroblast proliferation in vitro
IL 1	• Macrophages • T-lymphocytes	• Stimulates angiogenesis • Stimulates fibroblast proliferation in vitro • Stimulates fibroblast secretion of M-CSF • May inhibit collagen production
IL 6	• Macrophages • T-lymphocytes	• Detected in sponges in wounds • May be involved in cell recruitment and activation • Does not affect human Tenon fibroblast proliferation
IL 4	• T-lymphocytes	• Chemotactic to fibroblasts • Stimulates collagen and fibronectin synthesis
Interferons	• T-lymphocytes	• Inhibits collagen synthesis
bFGF	• Macrophages • Endothelial cells	• Stimulates fibroblast migration, proliferation, ECM synthesis, and contraction • Stimulates angiogenesis • Stimulates epithelial cell migration and ECM synthesis

inflammatory cells and fibroblasts and inducing them to release profibrogenic cytokines such as TGFβ, whereas fibroblasts are probably the main secretors of TGFβ, which exerts its effects by acting directly on fibroblasts.

Our laboratory and others have found TGFβ to be potently profibrogenic in conjunctival wound healing. It appears to be a potent inducer of human Tenon's fibroblast proliferation, migration, and collagen production, as well as stimulating angiogenesis and acting as a chemoattractant to other macrophages and fibroblasts.[138,194,201,204,246] TGFβ stimulates collagen production by increasing fibroblast expression of mRNA for collagen types I and III.[243] In contrast to PDGF, TGFβ increases granulation tissue formation by selectively inducing increased maturation of collagen bundles.[190]

All three isoforms (TGF-β1, -β2, -β3) are expressed by human conjunctival fibroblasts in vitro, but only TGF-β2 has been identified in vivo in unwounded human conjunctival stroma.[76,180] Using a pig skin wound healing model, Levine et al showed TGF-β2 and -β3 staining in fibroblasts and the inflammatory infiltrate associated with the healing dermis.[155] Recent research by ourselves has suggested that all 3 TGFβ isoforms appear to have similar actions in stimulating scarring.[65,66] This is contrary to Shah et al and others, who showed that TGF-β1 and -β2 promoted scarring in rodent wound healing, whereas TGF-β3 inhibited it.[219,220] These somewhat

contrasting results may well be attributable to differences between species, the wound healing models used, and the anatomical site studied, although it has been suggested that TGF-β2 may not be involved in human conjunctival scarring at all, as it is present to a similar degree in the aqueous of both nonglaucomatous and successfully filtered-glaucoma patients.[88] However, this viewpoint is somewhat in opposition with most of the literature that suggests that TGFβ is a profibrogenic cytokine.[234]

TGFβ expression is low during fetal scarless wound repair; however, if its level is artificially increased in such wounds, it provokes an inflammatory infiltrate associated with scar formation.[145] The addition of TGFβ will accelerate the healing of several types of wounds.[29,168,198] Its application increases the collagen content and breaking strength of normal wounds and steroid and adriamycin-impaired wounds.[149,186] The addition of antibodies against TGFβ reduces scarring after experimental glaucoma filtration surgery, and recently our laboratory has started clinical trials to test the efficacy of anti-human TGFβ antibody to inhibit conjunctival scarring.[64,218–220]

B. OTHER IMPORTANT CYTOKINES IN WOUND HEALING

Ford et al measured certain cytokine levels in implanted sponges collected from healing mouse skin wounds, and reported that significantly higher levels of macrophage-produced IL1, TNFα, IL6 and M-CSF were detected, compared to basal serum levels.[101] However, cytokines produced by lymphocytes, such as IL2, IL3, and IL4, were not detected. The failure to detect these cytokines is surprising, but could be explained by the possible insensitivity of the tests used or perhaps because the relevant cytokines were not investigated, as, clearly, lymphocytes do in some way participate in wound healing. Although the presence of certain T cell cytokines has not been demonstrated in vivo, Postlethwaite et al has shown that IL4 was chemotactic to fibroblasts and stimulated increased collagen and fibronectin production in vitro.[193,195]

Highest levels of mRNA for IL1 and TNFα exist at about 12–24 hours after wounding in a mouse model.[125] IL1 is an early proinflammatory cytokine, which facilitates neutrophil and monocyte entry into the wound by increasing their adhesion to vascular endothelial cells and also stimulates angiogenesis.[40,135] In addition, IL1 may have a catabolic role during the early stages of wound healing, first, by increasing collagenase expression and second, because it has been shown to inhibit collagen synthesis when added to lattice cultures of human skin fibroblasts.[112] Its effects on fibroblast proliferation are less well defined; it stimulated in vitro human Tenon's and dermal fibroblast proliferation, but had no effect on rat skin wound healing when administered subcutaneously.[70,147,232]

TNFα is also a proinflammatory cytokine, and some, but not all, research suggests that it may be able to directly stimulate fibroblast activity.[94] It increases human Tenon's and diploid fibroblast proliferation, induces angiogenesis, and stimulates monocytes to secrete M-CSF.[70,150,179,236] Like IL1, TNFα increased collagen synthesis only in serum-deprived fibroblasts. However, it increased glycosaminoglycans synthesis and collagenase production by fibroblasts cultured in both serum-deprived and serum-containing medium, suggesting a possible role for it in early wound catabolism.[84]

IL6 is a multifunctional cytokine produced by macrophages and some activated T cells. It appears to have different effects, depending on the target cell; Cunliffe et al found that IL6 did not stimulate proliferation in human Tenon's fibroblasts in vitro.[70] It did not stimulate proliferation of fibroblasts derived from sarcoid lungs, but it did when fibroblasts derived from lungs with diffuse interstitial fibrosis were used.[221] M-CSF appears to regulate several macrophage functions, including phagocytic activity, superoxide production and chemotaxis.[30,240,245]

The interferons are a heterogenous group of cytokines consisting of the Type I interferons (alpha- and beta-interferon) and Type II interferon (gamma-interferon). Alpha-interferon and beta-interferon are structurally dissimilar but are both involved in inhibiting viral infections. Gamma-interferon is produced by activated T cells and is involved in activating macrophages and promoting T cell differentiation during an immune response.

The interferons are thought to be equally effective in reducing wound healing by inhibiting collagen synthesis without affecting fibroblast proliferation.[83,131,148,170] Gamma-interferon decreases collagen types I and III mRNA expression in fibroblasts derived from patients with the fibrosing disease scleroderma; it has successfully improved clinical parameters, such as skin score and range of limb motion, in scleroderma patients in a small prospective, non-randomized trial.[132,203] Also, intralesional injections of alpha-interferon have reduced keloidal scarring.[35]

There has been only one ophthalmic clinical trial to date to test the beneficial effects of intraoperative and postoperative subconjunctival injections of alpha-interferon in reducing wound healing after glaucoma filtration surgery.[113–115] The success rate was 79% after 2 years follow-up, which was not significantly different to a success rate of 89% when 5-flourouracil was used. Alpha-IFN did not appear to offer any advantages over 5-flourouracil in terms of better intraocular pressure control and fewer side effects.

TGFα, which is produced by macrophages, stimulates epithelial cell, fibroblast, and endothelial cell mitogenesis and angiogenesis.[33] EGF, also secreted by macrophages, may stimulate fibroblast and epithelial cell chemotaxis and increase fibronectin production, which provides the initial scaffolding for clot formation during wound healing.[3,172] TGFα appears to be a more potent angiogenic factor than EGF. IGF-I, produced by both macrophages and lymphocytes, stimulates fibroblast and endothelial cell mitogenesis.[33] Finally, FGF-2, also secreted by macrophages, stimulates fibroblast and endothelial cell mitogenesis, and both FGF-2 (basic) and VEGF stimulate angiogenesis.[224]

It is important to remember that the wound healing response is a dynamic process, with cytokines appearing and disappearing at different time-points. This may provide difficulties in identifying and understanding the wound healing effects of individual cytokines. Furthermore, it is also important to take into account the possible interactions of the multiple cytokines in the wound healing milieu.[91] The study of their combined effects is certainly one of the challenges of future research.

IV. The Immune System in Persistent Conjunctival Scarring

A. POSSIBLE MECHANISMS OF PATHOGENESIS

Changes in the microenvironment might be able to affect the progression of wound healing, and, indeed, dysfunction of the immune response might profoundly modulate the quality and degree of wound repair. Fig. 4 represents the important balance required to ensure an appropriate wound healing response.

There is considerable evidence to suggest that abnormal inflammatory cell/fibroblast interactions, particularly those involving T lymphocytes and fibroblasts, might be involved in the pathogenesis of several fibrotic diseases and persistent conjunctival scarring. In a histological study of keloid scars, Martin et al showed that a leukocytic and fibroblast infiltrate could persist for up to several years in these lesions.[160] T cell/fibroblast interactions have also been implicated in fibrotic diseases, such as idiopathic pulmonary fibrosis and scleroderma. Lung T cells taken from patients with idiopathic pulmonary fibrosis stimulate human lung fibroblasts to increase their collagen production, although they also inhibit fibroblast proliferation.[216] In the bleomycin-induced model of pulmonary fibrosis, increased staining of macrophages for TGFβ is associated with increased collagen production.[137] Again, in scleroderma, tissues are infiltrated with mononuclear inflammatory cells, which express increased amounts of TGFβ.[206]

Hitchings and Grierson demonstrated that early trabeculectomy failures were associated with a marked inflammatory reaction and increased fibroblast numbers (Fig. 4).[122] In contrast though, late failures look quite different histopathologically, with a bleb wall of microkeloid appearance, consisting of fibrous tissue and only occasional mononuclear inflammatory cells.[2] In addition, immunostaining of conjunctival biopsies taken from repeat trabeculectomy patients who have failed their first trabeculectomies at 3 months suggests that lymphocytes are increased in number and that more are activated, staining positively for IL2.[174] Furthermore, we know that the application of topical steroids, by downregulating the immune system and reducing the number of inflammatory cells, is associated with reduced

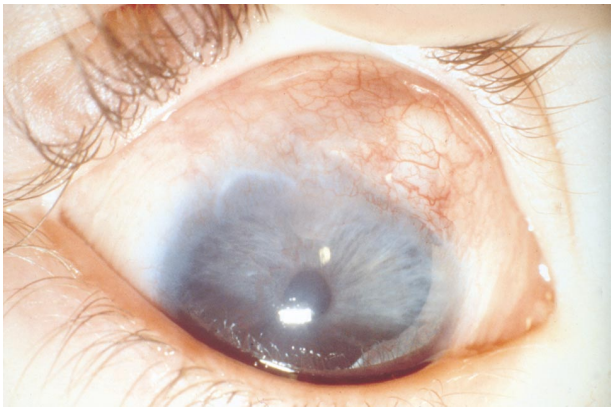

Fig. 4. An inflamed filtration bleb is predisposed to scar more aggressively and has a higher risk of failing filtration surgery. The cellular mechanisms that promote the development of this proinflammatory environment are only partially understood.

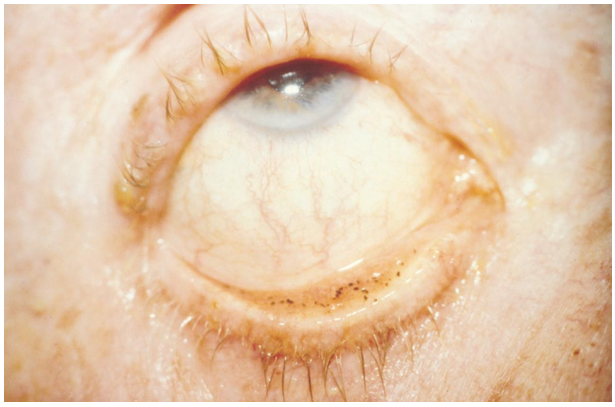

Fig. 5. This conjunctiva has developed adenochrome deposits following the use of adrenaline drops. Topical antiglaucoma medications can adversely affect the success rate for glaucoma filtration surgery. The use of multiple medications (β-blockers, pilocarpine and sympathomimetics) reduces the surgical success rate to 45%.

TABLE 2

Evidence for the Importance of Inflammatory Cells in the Pathogenesis of Excessive Conjunctival Scarring

Evidence	Findings
Fetal wound repair	• Normal wound healing: no inflammatory reaction and no scar tissue formation. • Addition of TGFβ and PDGF→inflammatory infiltrate and scarring[4,145]
Depletion studies	• Systemic steroids and anti-macrophage serum produce monocytopenia→reduced wound healing[67,127,151,152,169]
Effects of systemic drugs	• Vitamin A, arginine, growth hormone increase T cell function and increase wound healing[22,86,100]
Depletion studies	• Thymectomy→decrease in induction of suppressor T cells→increased wound healing[25] • Depletion of suppressor/cytotoxic T cell subset→increased wound healing[183]
Other fibrotic conditions of body	• Keloid lesions: increased lymphocytes and fibroblasts[160] • Lymphocyte infiltration of tissues from patients with idiopathic pulmonary fibrosis and scleroderma, and increased expression of TGFβ[137,216]
Fibrosing conjunctival diseases	• OCP and DICC: Increased macrophages, increased CD4 and CD8 T cells, increased MHC II expression[200] • Increased TGFβ, PDGF, TNFα expression[90]
Excessive conjunctival wound healing post filtration surgery	• Association between early trabeculectomy failures and marked inflammatory postoperative reaction[122] • Trabeculectomy failures at 3 months contain increased CD4 and CD8 T cells[174] • Multiple, > 3 years glaucoma drop use increases the no. macrophages and lymphocytes in conjunctiva of patients who scar aggressively post-operatively[48,49] • High risk for scarring patients (uveitic and black patients) contain increased macrophages, lymphocytes and fibroblasts[47]
Pharmacological modulation of inflammatory cells	• Topical steroids increase trabeculectomy success rates[12] • May directly modulate fibroblast function[235]

conjunctival wound healing and significantly better trabeculectomy success rates.[230] Therefore, it seems reasonable to suggest that overactivity of the immune system may play an important role in the pathogenesis of at least early trabeculectomy failures. Table 2 displays the evidence for the importance of inflammatory cells in the pathogenesis of excessive conjunctival scarring.

B. PREDISPOSING FACTORS

1. Previous Topical Drug Use

What factors might stimulate the immune system to promote persistent scarring? The chronic medical treatment that most glaucoma patients take before undergoing filtration surgery may make a significant contribution by changing the cellular composition of the conjunctiva (Fig. 5).[42,212,222] Broadway et al found that patients who had used multiple eye-drops for more than 3 years appeared to have significantly altered conjunctival cell profiles, showing an increase in the number of inflammatory cells and fibroblasts.[48] A few studies disagree with these findings; Baun et al found that the conjunctival cell profile of glaucoma patients did not change after 4 years of medical therapy, and Smith et al , using a rabbit model, found the same.[28,227] In addition, Gwynn et al did not find any difference in the number of inflammatory cells and fibroblasts between glaucoma patients who were better controlled after filtration surgery than those who were not so well controlled.[119] However, Broadway's research is important because it demonstrated that these changes were associated with an increased risk of aggressive scarring and failed glaucoma filtration surgery (Fig. 6).[49]

Research suggests that the irritating effects of these drops might stimulate a chronic inflammatory reaction predisposing to persistent scarring.[31] The active compounds themselves do not appear to directly stimulate fibroblast proliferation,[69,244] but studies have shown that the topical application of just the preservatives used in eyedrops can induce a considerable inflammatory reaction.[31] The preservatives in glaucoma medications seem to be able to provoke quite a toxic reaction; with increased expression of the inflammatory cell marker, HLA-DR, and apoptotic markers, Fas, FasL, and APO2.7, even in the absence of overt clinical inflammation.[26,27,44,77] Therefore, chronic use of antiglaucoma drops may somehow be able to stimulate a persistent inflammatory response in the conjunctiva of these patients, which may then predispose them to overproduce proinflammatory and profibrogenic cytokines when they are wounded. Clinically, such a process may be associated with a red eye. Such a process could ultimately affect the chances of successful glaucoma filtration surgery.

From research into the ocular fibrosing diseases,

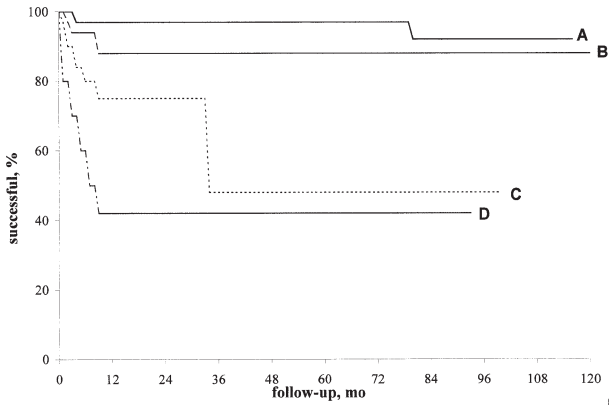

Fig. 6. Multiple topical glaucoma therapy and the success of trabeculectomy surgery. Kaplan-Meier survival analysis for glaucoma patients who underwent trabeculectomy, stratified by topical therapy regimes (Reprinted from Broadway DC et al[49] with permission of the authors and the American Medical Association [Copyright 1994, American Medical Association]). A = minimal therapy, B = β-blockers, C = β-blockers + miotic, D = β-blockers + miotic + sympathomimetic.

we have also learned a great deal about the possible role of the immune system in the pathogenesis of aggressive conjunctival scarring, such as ocular cicatricial pemphigoid and drug-induced conjunctival cicatrization.[37,45,88] The main morphological features in ocular cicatricial pemphigoid are the infiltration of the substantia propria by inflammatory cells, followed by fibrosis of the substantia propria by new connective tissue.[10,36,103,207] CD8+ and CD4+ T cell numbers are increased, as well as neutrophils and macrophages, with increased MHC II expression by both fibroblasts and macrophages.[39,200] TGFβ appears to be an important cytokine as its expression is increased, and greater disease activity and progression seem to be associated with increased conjunctival inflammation.[38,89,90] The pathological changes in drug-induced conjunctival cicatrization are similar to those found in ocular cicitricial pemphigoid.[182,197] Again, the profibrogenic cytokines implicated in this condition appear to be TGFβ, PDGF, bFGF and TNFα, which, as has been mentioned before, are all produced by inflammatory cells.

2. Other High Risk Patients

There are fewer studies that examine the immune system's contribution to aggressive conjunctival scarring in other high-risk patients. Some studies, but not all, suggest that the conjunctiva of Afro-Caribbean and uveitic patients may contain an increased number of macrophages, lymphocytes, and fibroblasts.[46,47,162] Differences in healing depending on age are largely unsubstantiated, although recent research with a murine incisional model suggests that delayed entry of inflammatory cells and decreased expression of TGFβ with age may affect the quality and degree of wound healing.[13–15] The conjunctiva of younger patients does not appear to show a change in inflammatory cell counts.[1,56,118] These studies were generally small in number and did not always look at the very young age groups. Differences in conjunctival wound healing according to age have largely been unstudied.

V. The Resolution of the Inflammatory Phase in Wound Healing

Importantly, as the wound healing response resolves, it is necessary for the immune system to deactivate itself and reduce cell numbers in order to restore immune homeostasis, since a persistent, hugely expanded population of activated inflammatory cells could be quite damaging.[8] This, therefore, involves the death of large numbers of cells that had originally entered and proliferated at the wound site. It is thought that at the peak of the immune response, T cell death occurs through a process called activation-induced cell death or Fas-mediated apoptosis. Apoptosis is a gene-directed process whereby a cell induces its own death. Apoptotic cells have a characteristic morphology, with condensation of the nucleus and cytoplasm and nuclear fragmentation within an intact cell membrane. This form of cell death does not result in the release of the cell contents into the exterior, which might otherwise excite an inflammatory response. Fas-mediated apoptosis consists of interactions between activated T cells mediated by their surface molecules, FasL, and its receptor, Fas.[50,80] These interactions result in a cascade of specific enzymatic reactions, which ultimately result in the programmed death of these T cells. Toward the end of the immune response, T cell numbers are reduced by nutrient or cytokine deprivation-mediated apoptosis and, although the initiating event is different, the mechanism of death is similar.[7]

Therefore, if the immune response fails to resolve properly, it is conceivable that persistently activated inflammatory cells might continue to secrete profibrogenic cytokines and promote the development of an aggressive scarring reaction. It is now known that stromal cells, including in vitro fibroblasts and endothelial and epithelial cells, can prevent cytokine-deprivation mediated T cell apoptosis.[116,128]

Indeed, excessive fibroblast-mediated T cell survival seems to be a feature of chronic inflammatory conditions, such as eczema and rheumatoid arthritis.[191,209] Recently, our group and others have identified that fibroblasts produce the soluble factor, interferon-beta, which is responsible for preventing cytokine-deprivation mediated T cell death.[159] It has also been suggested that TGFβ may prevent Fas-

mediated T cell apoptosis.[54,109] It also seems that intraocular levels of TGFβ can be correlated with the amount of scarring induced in fibrotic diseases like proliferative vitreoretinopathy.[63] We could therefore speculate that in persistent conjunctival scarring, an abnormal cycle of interactions between T cells and fibroblasts might be set up, with the fibroblasts keeping the T lymphocytes alive, which, in turn, stimulate the fibroblasts to continue to produce scar tissue. In terms of aggressive conjunctival wound healing, this is an area of research requiring further evaluation.

VI. The Role of the Immune System in Other Ocular Fibrosing Diseases

Although this review concentrates on the impact of the immune system on conjunctival wound healing, it is important to note that interactions between inflammatory cells and fibroblasts may play a role in other ocular inflammatory or wound healing disorders. The pathophysiology of proliferative vitreoretinopathy can be compared to a wound healing reaction in a specialized tissue. Several studies have shown that inflammatory cells e.g., macrophages and lymphocytes together with fibroblastic-like retinal pigment epithelial (RPE) cells, make up the periretinal membranes.[57,133] Some studies have suggested that the macrophages seem to be of the acute inflammatory subtype when the intraocular proliferation is more severe and that the lymphocytes present in proliferative vitreoretinopathy tissue are activated and belong to both subsets.[57,58,93]

It is likely that the growth factors secreted by these inflammatory cells contribute to the pathogenesis of PVR; IL1, IL6, TNFα, IFNγ, TGFβ, and PDGF have all been identified in samples of PVR vitreous or membranes.[157] Limb et al also found that there was significant HLA-DR expression in proliferative vitreoretinopathy membranes and suggested that local autoimmune responses may be occurring within them.[156] The body of evidence strongly suggests that key interactions between inflammatory cells and fibroblastic-like RPE cells contribute significantly to the pathogenesis of the disease.

The chronicity of the autoimmune disorder thyroid eye disease may also be caused by fibroblast/T cell interactions. Although the nature of the autoantigen initiating the disease is not well characterized, it is known that the associated inflammatory infiltrate releases a combination of cytokines, which stimulate orbital fibroblasts to produce glycosaminoglycans.[18,120] It would be interesting to examine whether orbital fibroblast-mediated prevention of T cell apoptosis might contribute to the chronic inflammatory process of thyroid eye disease. Finally, recent evidence suggests that macrophages participate in corneal wound healing after excimer laser keratectomy.[175] It has been suggested that their ability to influence the development of corneal haze should be examined.

VII. The Modulation of the Immune System in Wound Healing

A. CURRENT THERAPIES

The success rate of glaucoma filtration surgery may be optimized through the use of a combination of pharmacological agents that modulate the inflammatory and proliferative phases of the wound healing reaction.[86,215,248] The most commonly clinically used drugs are corticosteroids and the antimetabolites, 5-fluorouracil (5-FU) and mitomycin-C (MMC), which improve trabeculectomy success rates in both low and high risk for scarring patients.[11,59] Topical antiprostaglandins, which are known inhibitors of inflammation, have not proved to be useful in improving the outcome of fistulizing surgery, although newer agents have not been tested.[163]

Trabeculectomy surgery is more successful with postoperative steroids, and systemic steroids do not appear to have an advantage over topical administration.[12,202] Other studies have examined the effect of steroids on reducing bleb inflammation and fibrosis.[110,167]

Corticosteroids have potent antiinflammatory and immunoregulatory effects.[71] The mechanisms of their effects are still not entirely understood. When taken systemically, corticosteroids cause a redistribution of circulating peripheral blood lymphocytes to the bone marrow, resulting in a lymphocytopenia and monocytopenia.[95] Steroids can augment suppressor T cell activity and inhibit T cell proliferation and antigen presentation.[213] Monocytes may be especially sensitive to steroids, with suppression of bactericidal activity. They also appear to inhibit the access of neutrophils and monocytes to the inflammatory site.[96] Steroids reduce vascular permeability and may decrease the secretion of proinflammatory cytokines. Dexamethasone-treated full-thickness skin wounds in mice showed significantly reduced expression of mRNA for IL1α, IL1β and TNFα.[125,210] Therefore, topically applied steroids probably exert most of their effects by decreasing the inflammatory phase of conjunctival wound healing, by reducing the influx of inflammatory cells and by decreasing the production of pro-fibrogenic cytokines.

Some authors have suggested that steroids may affect fibroblast function directly.[19,82,235] They have been shown to inhibit the in vitro contraction of collagen gels, and they appear to have a biphasic effect on fibroblast proliferation, inhibiting proliferation at low doses and stimulating it at higher doses.[41,74] Clinically, steroid use has been reportedly associated

with a change in the morphology of filtration blebs, causing thin, cystic bleb walls and even necrosis.[111,167,205,231] Miller et al found that topical steroids had only a temporary delaying effect on fibroblast proliferation in a rabbit model of glaucoma fistulizing surgery. The main effects appeared to be caused by a reduction in the number of inflammatory cells and a decrease in aqueous chamber flare, reflecting the stabilization of the blood-aqueous barrier.[165] These somewhat different findings may be because the rabbit displays more aggressive scarring, and, therefore, further research into the effects of steroids on human conjunctival wound healing would be of interest.

Mitomycin-C and 5-FU are chemotherapeutic agents, which exert their effects on malignant cells by inducing cell death or apoptosis. Mitomycin-C acts on cells by damaging their DNA, through cross-linking bases in the same or adjacent DNA strands. 5-FU acts on proliferating cells primarily by inhibiting thymidylate synthetase and preventing the synthesis of DNA. With respect to inhibiting conjunctival scarring, our laboratory has shown that these agents induce human Tenon's fibroblast apoptosis, as well as inhibiting fibroblast proliferation, migration, and collagen contraction.[34,68,139,140,176] Weinreb suggested that 5-FU could have a beneficial effect on inflammation; he reported that some of his patients with inflammatory glaucomas required less steroid after surgery than before.[242] Although these drugs are primarily used in trabeculectomy surgery because of their actions on fibroblast function, they almost certainly exert the same effects on inflammatory cells, as shown by their virtual absence in biopsies taken from Mitomycin-C-treated blebs.[181,223]

The use of these drugs is not entirely ideal, because they do have potential side effects. Corticosteroids may be associated with cataractogenesis, raised intraocular pressure, and infection. Hypotony, endophthalmitis, and toxic effects on the corneal epithelium are complications associated with antimetabolite use. Furthermore, it is not completely understood yet why some patients at high risk for scarring still fail filtration surgery, despite the use of these drugs.[61,226] One reason may be because growth-arrested human Tenon's fibroblasts, when tested in vitro, are still able to migrate and secrete certain growth factors, such as TGFβ. This may allow some patients to overcome the antiscarring effects of the antimetabolites.[177]

B. FUTURE THERAPIES

The antimetabolites and steroids have certainly had a significant impact on improving the success rate of filtration surgery. However, future treatments could consist of more targeted therapy that would be directed at blocking specific cytokines or interfering with important inflammatory cell/fibroblast interactions that might lead to persistent scarring.

As mentioned before, this approach is already being investigated. Gillies et al found that postoperative subconjunctival injections of alpha-interferon compared similarly to 5-FU in terms of reducing wound healing and controlling intraocular pressure after 2 years of follow-up. Unfortunately, combination therapy did not appear to have an increased effect.[114] Research in our laboratory is currently in progress to investigate the effects of antihuman TGFβ antibody in reducing conjunctival wound healing.[64]

Cyclosporin is a powerful immunosuppressive agent most commonly used in preventing organ transplant rejection. In ophthalmology it may be of clinical benefit in the treatment of T cell-mediated chronic inflammatory conditions such as vernal and atopic keratoconjunctivitis.[123,214] It exerts its effects by blocking the proliferation and activation of T cells by inhibiting IL2 transcription and the expression of the receptor for IL2.[104,144] In atopic keratoconjunctivitis, it reduces the inflammatory infiltrate, normalizes the CD4:CD8 ratio, and reduces IL2, gamma-IFN, and HLA-DR expression.[121] It may also be able to modulate wound healing, and encouraging data have been reported in a rabbit wound healing model.[74,173,184] However, it appears to have no effect on corneal epithelial and rat skin wound healing, and it may stimulate TGFβ and induce gingival overgrowth.[6,87,98,143,192] Therefore, the role of cyclosporin in modulating excessive conjunctival scarring needs to be evaluated further. It could be useful in modulating the inflammatory cell profile of high-risk patients preoperatively or to reduce the inflammatory condition of those patients with severe chronic inflammatory conjunctivitis preoperatively. Alternatively, it could be an important steroid sparing agent for those patients at high-risk for scarring who are intolerant of intensive topical steroids.

Some recent research has highlighted the potential role of mast cells in wound healing.[154] They are present in hypertrophic scars, and one of the secretory products, histamine, may be involved in promoting in vitro wound closure.[141,146,153] Disodium cromoglycate has been shown to reduce skin wound healing and collagen synthesis in a rat wound healing model.[73] Another secretory product, heparin, has been shown to be necessary for stabilizing other secretory granules inside the mast cells.[102,126] In addition, it appears to inhibit human corneal fibroblast proliferation in vitro.[78] Recently, we have shown that the interoperative combination of 5FU and heparin significantly reduces the risk of postoperative retinal scarring in patients undergoing retinal detachment surgery, and it may decrease the risk of the development of proliferative retinopathy (Chang L et al: *Ophthalmology*, in press).

Another immunomodulatory approach to wound healing might be the use of cytokines, which could play some role in the development of ocular immune privilege. This phenomenon involves a numbers of factors that protect the interior of the eye from the damaging inflammatory effects accompanying immune responses. One novel cytokine that has been implicated is IL10.[72,97]

In conclusion, many of the constituents of the immune system participate in the wound healing response. It is evident that a fine balance in the immune system's response to wounding is required to ensure the correct degree of repair. The challenge will be to develop specific therapies to switch wound healing on or off depending on the clinical response of the patient, to avoid some of the unsatisfactory side-effects of the drugs currently used, and to ultimately improve the surgical success rate of glaucoma filtration surgery.

Method of Literature Search

A comprehensive international literature search was achieved through the use of Medline, Winspurs, and PubMed—databases for basic science and clinical medical research—as well as obtaining articles cited by authors but not found on these databases. Search words included: *wound healing, conjunctiva, immune system, T-lymphocyte, macrophage, glaucoma, trabeculectomy.* These were used in combinations to ensure that an exhaustive selection of the relevant references was obtained. The databases dated from 1960–1999.

Searches on the databases dated from 1960. Older references were obtained from checking older articles.

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